UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1

2 **AD-A240 997**

2

1b. RESTRICTIVE MARKINGS

3. DISTRIBUTION/AVAILABILITY OF REPORT
Approved for public release; distribution is unlimited

4. PERFORMING ORGANIZATION REPORT NUMBER(S)
NMRI 91-58

5. MONITORING ORGANIZATION REPORT NUMBER(S)

6a. NAME OF PERFORMING ORGANIZATION	6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
Naval Medical Research Institute		Naval Medical Command

6c. ADDRESS (City, State, and ZIP Code)	7b. ADDRESS (City, State, and ZIP Code)
8901 Wisconsin Avenue Bethesda, MD 20889-5055	Department of the Navy Washington, DC 20372-5120

8a. NAME OF FUNDING/SPONSORING ORGANIZATION	8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
Naval Medical Research & Development Command		

8c. ADDRESS (City, State, and ZIP Code)	10. SOURCE OF FUNDING NUMBERS			
	PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
8901 Wisconsin Avenue Bethesda, MD 20889-5044	62233N	M33C30.005	1051	DN249507

11. TITLE (Include Security Classification)
Variations in thymocyte susceptibility to clonal deletion during ontogeny: implications for neonatal tolerance

12. PERSONAL AUTHOR(S) Zacharchuk CM, Mercep M, June CH, Weissman AM, Ashwell JD

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
journal article	FROM _____ TO _____	1991	6

16. SUPPLEMENTARY NOTATION
Reprinted from: The Journal of Immunology 1991 July 15 Vol. 147 No.2 pp. 460-465

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	T cell development; thymic development; calcium; T cell receptor, Repertoire

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Phyllis Blum, Librarian.	(301) 295-2188	MRL/NMRI

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

0022-1767/91/1472-0460$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 147, 460-465, No. 2, July 15, 1991
Printed in U.S.A.

VARIATIONS IN THYMOCYTE SUSCEPTIBILITY TO CLONAL DELETION DURING ONTOGENY

Implications for Neonatal Tolerance

CHARLES M. ZACHARCHUK,* MLADEN MERCEP,* CARL H. JUNE,† ALLAN M. WEISSMAN,‡ AND JONATHAN D. ASHWELL[1]*

*From the *Biological Response Modifiers Program, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, †Immune Cell Biology Program, Naval Medical Research Institute, Bethesda, MD 20889, and ‡Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892*

Activation of immature thymocytes via the TCR results in programmed cell death and clonal deletion. We have examined thymocytes from mice of different ages and observed that, whereas TCR-mediated signaling caused deletion of thymocytes from newborn and 3-week-old mice, it failed to delete thymocytes from mice of 1 week of age. This could not be attributed to differences in cell surface TCR expression, TCR-mediated phosphoinositide hydrolysis or Ca^{2+} mobilization, or total cellular levels of TCR ζ- and η-chains. Moreover, thymocytes of all ages were equally susceptible to corticosteroid- and Ca^{2+} ionophore-induced programmed cell death. These data are consistent with the notion that fetal and neonatal thymocytes represent a relatively synchronous wave of cells passing through phases in which they are susceptible and then resistant to TCR-induced programmed cell death. They also support the notion that the classical phenomenon of neonatal tolerance is due to clonal deletion and that the inability of allogeneic cells to tolerize mice at 1 week of age is because the thymocytes are refractory to TCR-$\alpha\beta$-mediated clonal deletion.

For mature T cells, the predominant outcomes of TCR occupancy include secretion of lymphokines and entry into the growth cycle. For many thymocytes, however, receptor occupancy leads to clonal inactivation. This process, known as negative selection, is fundamental to the establishment of immune self-tolerance. Two types of inactivation have been observed, clonal anergy and clonal deletion. Clonal anergy occurs when potentially autoreactive cells persist but fail to respond to self-Ag by secreting lymphokines and proliferating (1-3). Clonal deletion is the active elimination of the autoreactive cells and, in several experimental systems using thymocytes, has been shown to be the result of programmed cell death (apoptosis) (4-6). Some of the hallmarks of activation-induced T cell programmed cell death are a requirement for extracellular Ca^{2+} and new mRNA and protein synthesis, the autofragmentation of nuclear DNA into 180- to 200-bp multimers, and prevention of the process by cyclosporine A (7-9).

Neonatal tolerance, a classical model of Ag-specific tolerance, is induced by injection of viable allogeneic cells into newborn mice (10). If this is done within the first day of life, the recipients fail to reject skin grafts from the allogeneic mice weeks or even months later (i.e., are tolerant). Inoculation of older mice is progressively less effective. More recently, it has been shown that injection of anti-TCR antibodies into young adult mice results in thymocyte deletion by the process of programmed cell death (9). In the present study, anti-TCR antibodies or the SEB[2] "superantigen" were used to activate thymocytes from mice of different ages, in an attempt to integrate the two experimental models. Surprisingly, although no difference in TCR-initiated signal transduction was detected, the manifestation of clonal deletion varied in a distinctive fashion as a function of ontogeny. The results support the notion that the susceptibility of the "immature" thymocyte subpopulation to clonal deletion changes during the course of development and provide a cellular model for the phenomenon of neonatal tolerance.

MATERIALS AND METHODS

Mice. Timed pregnant B6 mice were obtained from the Frederick Cancer Research Center (Frederick, MD).

Antibodies and reagents. Hamster IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). H57 is a hamster anti mouse TCR $\alpha\beta$ antibody (11), F23.1 is a mouse anti-Vβ8 antibody (12), RR-4-7 is a rat anti-mouse Vβ6 antibody (13), 2C11 is a hamster anti-mouse CD3-ϵ antibody (14), and OKT3 is a mouse anti human CD3-ϵ antibody (15). FITC-GK 1.5 (anti-CD4) (16) and biotinylated 2 43 (anti CD8) (17) were kindly provided by Ada Kruisbeek (National Institutes of Health, Bethesda, MD). SEB and DEX were purchased from Sigma Chemical Co. (St. Louis, MO). Ionomycin was purchased from Calbiochem (La Jolla, CA).

Thymocyte depletion assays. Mice were injected i.p. with control hamster IgG or H57 antibodies in PBS, using a fixed antibody/mouse weight ratio in a given experiment. Forty-eight hours later, the mice were sacrificed, their thymi were removed and made into single cell suspensions, and viability was determined by trypan blue exclusion. Percentage of recovery was expressed as the number of thymocytes in treated vs control animals:

$$\frac{\text{Average number of viable thymocytes recovered/treated mouse}}{\text{Average number of viable thymocytes recovered/hamster IgG-treated mouse}} \times 100$$

Received for publication January 14, 1991.
Accepted for publication April 15, 1991.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and requests for reprints to Jonathan D. Ashwell, Biological Response Modifiers Program, Building 10, Room 13N-268, NCI, NIH, Bethesda, MD 20892.

[2] Abbreviations used in this paper: SEB, Staphylococcal enterotoxin B; B6, C57BL/6; H57, H57 597; 2C11, 145-2C11; PI, phosphoinositide; DEX, dexamethasone; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

For some experiments, timed-pregnant B6 mice were used. Day 0 (fetal day 19, the day of birth) pups were injected within 24 h of birth. To determine the effects of SEB on Vβ expression in vitro, thymic lobes from mice of different ages were cultured in medium consisting of DMEM supplemented with 10% FCS, 2 mM glutamine, 10 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, 0.11 mg/ml sodium pyruvate, 5×10^{-5} M 2-ME, and 10 mM HEPES (complete medium), with or without SEB (30 μg/ml). The thymic lobes were then cultured on Nucleopore filters supported by Gelfoam gelatin sponges (18). After 24 h, the thymic lobes were removed, made into single-cell suspensions, cultured in complete medium for 4 to 8 h at 37°C as described (19), and then analyzed for Vβ expression by flow cytometry.

Flow cytometry. To determine CD3 and Vβ expression, 10^6 thymocytes were stained with FITC-conjugated H57 or culture supernatants (F23.1 and RR-4-7), followed by FITC-labeled goat anti-mouse or FITC-goat anti-rat Ig (Jackson ImmunoResearch Laboratories), and analyzed with a FACScan (Becton-Dickinson Immunocytometric Systems, Mountain View, CA) by using FACScan software. To determine CD3, CD4, and CD8 expression, thymocytes were stained with FITC-conjugated 2C11 or FITC-GK 1.5 plus biotinylated 2.43, followed by phycoerythrin-avidin (Calbiochem).

Measurement of phosphatidylinositol hydrolysis. Thymocytes were loaded with myo-[^{3}H]inositol (14 Ci/mmol; NEN, Boston, MA) for 3 h at 37°C, as described (20). The cells were thoroughly washed and divided into duplicate groups, at the indicated numbers, in tubes containing 10^6 LK 35.2 cells to provide FcR for antibody cross-linking (21). Activating anti-TCR antibodies were added, and at the indicated times the cells were lysed, lipids were extracted, and the water-soluble labeled products of PI hydrolysis were measured by anion exchange chromatography (20). The mean cpm achieved for each experimental point was divided by the total cpm incorporated by the cells (yielding percentage of labeled phospholipid), and then the percentage of labeled phospholipid generated in the absence of H57 (background) was subtracted, to yield change in percentage of labeled phospholipid.

Measurement of $[Ca^{2+}]_i$. Determination of thymocyte $[Ca^{2+}]_i$ was modified from the procedure of Rabinovitch and June (22). Briefly, thymocyte suspensions were prepared from B6 mice of the indicated ages and, after a 3-h culture at 37°C in complete medium, 5×10^6 cells/ml were loaded with 1.8 μM Indo-1 (Molecular Probes, Junction City, OR), in HBSS without phenol red, for 30 min at 30°C. For each sample, either HBSS (control) or 20 μg of H57 antibody were added to 5×10^5 cells in 0.1 ml of HBSS on ice for several min; cells were washed once with HBSS and added to 0.5 ml of prewarmed 37°C HBSS containing 50 μg/ml goat anti-hamster IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Cells were then analyzed for violet/blue fluorescence emission ratio (395 nm/500 nm), as described.

Analysis of thymocyte viability by flow cytometry. Thymic lobes from B6 mice were removed on different days after birth and cultured as a single-cell suspension in 5-ml tubes (Falcon no. 2058; Becton Dickinson, Lincoln Park, NJ) at 2×10^6/ml, in complete medium, with or without the indicated reagents. After 15 h, cells were analyzed in the presence of propidium iodide (10 μg/ml) with a FACScan, by using an excitation wavelength of 488 nm and detection at 585 nm; 10,000 events were collected for each data point. The cells were easily resolved into positive and negative populations, and electronic gates were set accordingly. The data are expressed as the percentage of analyzed cells that did not stain with propidium iodide (viable). The numbers in parentheses represent (percentage of unstained cells in each treatment group/percentage of unstained cells in cultures with medium alone) × 100.

RESULTS

TCR mediated signal transduction at different days of development. Inoculation of newborn mice with allogeneic cells is superior to inoculation of 1-week-old mice in terms of the induction of long term tolerance (10). One possible explanation for this is that the signal transduction pathways are quantitatively or qualitatively different between thymocytes of varying ages. To test this possibility, early signal transduction events were assessed in thymocytes from mice of different ages. First, the hydrolysis of PI to inositol phosphates in response to TCR cross-linking by an anti-TCR-αβ mAb, H57, was examined in B6 thymocytes (Fig. 1). The kinetics of total inositol phosphate production by both fetal day 18, neonatal day 0, and day 7 thymocytes in the presence of $LiCl_2$ were similar (Fig. 1*A*). Furthermore, dose-response analysis of thymocytes from day 0 and day 7 revealed that, if anything, day 7 cells responded a bit better to stimulation with H57 (Fig. 1*B*). PI hydrolysis was slightly greater with thymocytes from 3-week-old mice (perhaps reflecting the accumulation of single-positive thymocytes), although the dose-response curves were identical. Second, H57-induced increases in $[Ca^{2+}]_i$ were measured. Two parameters were quantitated, the time course of the increase and the mean increase in $[Ca^{2+}]_i$. Thymocytes from day 0, 1 week, and 3 weeks behaved in an identical manner. The majority of cells in the three groups responded to H57 and did so with a unimodal increase in $[Ca^{2+}]_i$ (data not shown). All groups responded rapidly and with equal plateau values (Fig. 2). Thus, no significant differences were found in at least these two signal transduction pathways during ontogeny.

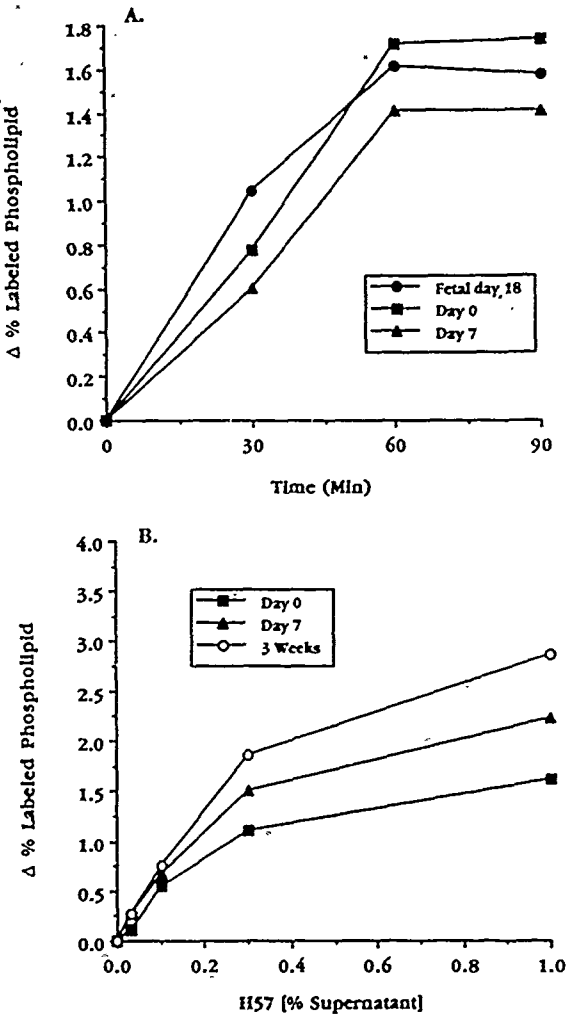

Figure 1 Thymocyte H57-induced PI hydrolysis as a function of age. Thymocytes from B6 mice, age fetal day 18, day 0, day 7, or 3 weeks, were loaded with myo-[^{3}H]inositol and divided into duplicate groups of 2×10^6 cells/point (*A*) or 3×10^6 cells/point (*B*). In tubes containing 10^6 LK 35.2 cells *A* a 1/50 dilution of H57 supernatant was added to the tubes at time 0 and at the indicated times the cells were lysed, lipids were extracted, and the water-soluble labeled products of PI hydrolysis were measured by anion exchange chromatography. *B*, the indicated concentrations of H57 supernatant were used to stimulate the thymocytes for 75 min, at which time the cells were handled as in *A*.

Injection of anti-TCR antibody and thymocyte recovery. A relatively late event, and one more directly relevant

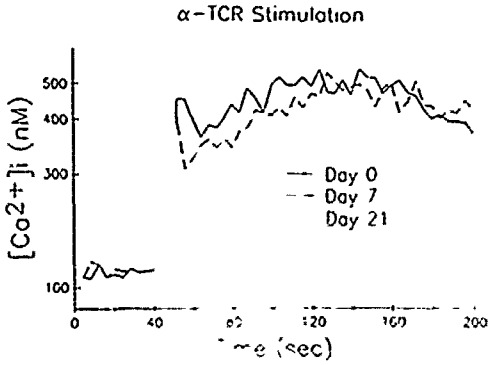

Figure 2. Thymocyte Ca^{2+} mobilization as a function of age. Determination of thymocyte $[Ca^{2+}]_i$ with Indo-1 was performed as described in *Materials and Methods*. The initial baseline $[Ca^{2+}]_i$ depicted on the *abscissa* represents cells that were loaded with Indo-1 but were incubated with HBSS, not H57, before the addition of the goat anti-hamster antibody. The curves after the gap reflect the $[Ca^{2+}]_i$ levels measured after placement of the antibody treated cells in the flow cytometer (which was done approximately 10 s after the addition of the goat anti-hamster antibody). Note that in this short time the cells from all groups responded at near maximal levels.

TABLE I

Thymocyte depletion by anti-αβ antibodies after birth[a]

Expt.	Thymocyte recovery (%)		
	Day 0 (n)	1 Week (n)	3 Weeks (n)
1	46 (4)	89 (3)	ND
2	56 (5)	110 (3)	ND
3	59 (8)	83 (5)	ND
4	ND	ND	37 (28)
5	ND	104 (5)	68 (5)
6	43 (5)	104 (3)	36 (3)
Mean	51	97	47

[a] B6 mice on day 0 (day of birth) or at 1 or 3 weeks after birth were injected i.p. with H57 (10 μg/g of mouse weight in experiments 1, 3, and 6; 5 μg/g of mouse weight in experiments 2, 4, and 5) or equivalent amounts of hamster IgG control antibody diluted in PBS. After 48 h, thymi were harvested, and viable thymocyte recovery was quantitated with trypan blue dye and light microscopy. *n*, number of mice in each group.

to the process of tolerance induction, is the activation-induced clonal deletion of thymocytes by apoptosis (5). To determine how this response might vary as a function of age, B6 mice were injected with H57 (which has been shown to delete a subset of $\alpha\beta^+$ immature thymocytes in fetal thymic organ culture) (19) or a hamster IgG control on day 0 (day of birth) or 1 week or 3 weeks after birth. Thymocytes were collected 48 h later and analyzed for viable cell recovery (Table I). Inoculation on the day of birth reproducibly resulted in about a 50% decrease in viable cell yield, whereas inoculation 7 days later had little effect. Interestingly, and in agreement with a previous report using the anti-CD3 antibody 2C11 (9), at 3 weeks of age the injection of H57 once more resulted in a substantial reduction in cell yield. In independent experiments, the injection of similar amounts of an anti-$H\text{-}2^b$ mAb had little effect upon thymocyte recovery in mice at any of these ages (data not shown).

The difference between the response of day 0 and day 7 mice could not be explained on the basis of surface TCR expression, because thymocyte cell surface TCR levels were similar between these two groups, except for a slightly greater number of TCR^{high} cells on day 7 (Fig. 3). Even in young adult mice, the large majority of thymocytes were TCR^{low} (there was no discernable difference in the TCR staining profiles between 3 and 6 weeks). Flow cytometric analysis of CD4, CD8, and CD3 expression of thymocytes from 3-week-old mice demonstrated that treatment with H57 caused preferential deletion of the $CD4^+$ $CD8^+$ (double-positive) TCR^{low} cells, with relative enrichment (1.5- to 2-fold) of the $CD4^+$ $CD8^-$ (single-positive) TCR^{high} cells (Fig. 4). Although not demonstrated well in this experiment, in other experiments $CD4^-$ $CD8^+$ cells were also enriched by this treatment. Note that, by taking absolute cell number into account when comparing the experimental and control groups, we found that anti-TCR treatment predominantly affected the double-positive cells. In three independent experiments, treatment with H57 caused a 60% decrease in total cell recovery. In these experiments, there was a 65% decrease in the absolute number of double-positive cells but only a 35% decrease in the number of single-positive cells. Given that double-positive cells are the precursors of single-positive cells, at least some of the decrease in the latter is likely due to fewer cells being available to enter the single-positive population, rather than due to a direct effect of anti-TCR antibody on the survival of single-positive cells. Whether the H57 treatment directly caused the loss of any single-positive cells is unknown. These results are in agreement with organ culture studies, in which anti-CD3 antibodies were also found to primarily kill double-positive thymocytes (5). In another fetal organ culture system, H57 was shown to delete ~50% of TCR^{low} thymocytes and virtually all TCR^{high} cells (19). The data in the present study are consistent with the notion that TCR^{high} cells are actually at least relatively resistant to direct deletion but, when antibody is administered early in organ culture, their appearance is prevented by deletion of their TCR^{low} precursors.

Treatment of thymocytes in organ culture with SEB. It was formally possible that the change in the susceptibility to deletion was not a property intrinsic to the thymocytes themselves but was somehow the result of changes in accessibility of the thymocytes [illegible] (i.e., across a putative blood-thymus barrier) or [illegible] macokinetics of injected H57. To avoid the potential complications that might be introduced by using antibodies in vivo, we made use of a "superantigen" that reacts with the majority of members of particular Vβ families and causes thymocyte programmed cell death and clonal deletion (6, 23). SEB is recognized by TCR that contain Vβ3, Vβ7, or Vβ8. Exposure of thymocytes to SEB in vivo or in vitro specifically deletes thymocytes bearing these TCR (24). Thymic lobes from day 0 or day 7 B6 mice were placed in tissue culture in the presence or absence of SEB. After overnight incubation, a cell suspension was made and expression of Vβ8 or Vβ6 was assessed by flow cytometry (Fig. 5). SEB caused a substantial decrease in the number of $V\beta8^+$ thymocytes when added to day 0 thymic lobes. As expected, there was a reciprocal increase in the fraction of SEB-resistant $V\beta6^+$ thymocytes. In contrast, SEB had little effect on the fraction of $V\beta8^+$ cells and caused virtually no increase in $V\beta6^+$ cells in day 7 thymic lobes. These results were reproducible, with similar results being obtained in three independent experiments (average of 37% reduction of $V\beta8^+$ thymocytes on day 0 and 6% reduction of $V\beta8^+$ thymocytes on day 7), as well as in experiments in which SEB was injected into B10.A mice (our unpublished data). These results, generated in an in vitro system with a superantigen, together

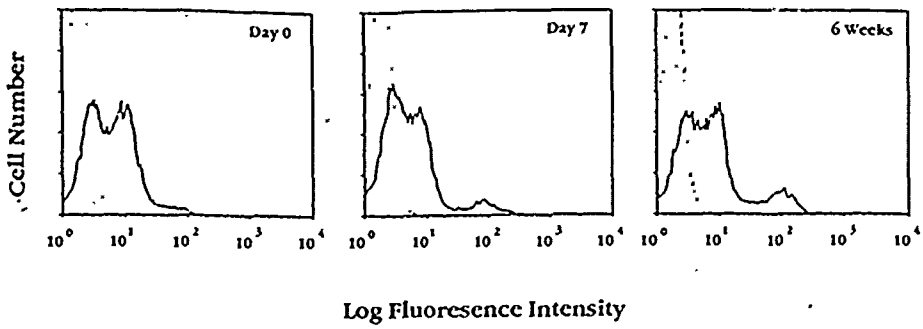

Figure 3. Thymocyte surface phenotype as a function of age. Thymocytes from B6 mice were harvested on day 0, day 7, or 6 weeks after birth, stained with either FITC-coupled OKT3 (anti-human CD3- · · · ·) or FITC-coupled H57 (——), and analyzed on a FACScan.

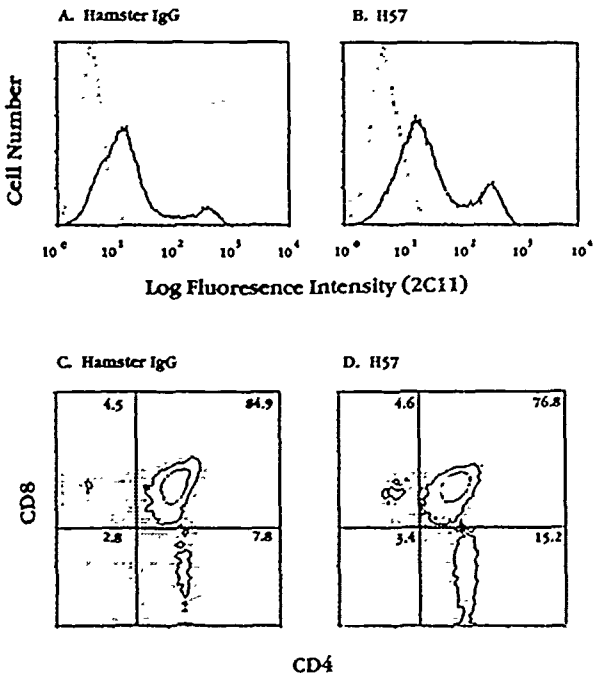

Figure 4. Depletion of immature thymocytes after in vivo treatment with H157. Three-week-old B6 mice were injected with 50 μg of hamster IgG (*A* and *C*) or H57 (*B* and *D*) i.p., and 48 h later thymi were removed, made into single-cell suspensions, and analyzed by flow cytometry for CD3 expression (*A* and *B*) (· · · ·, FITC-OKT3; ——, FITC-2C11) or CD4 and CD8 expression (*C* and *D*). Note that H57 and 2C11 do not cross-block one another when used for staining cells (our unpublished observations). The percentage of total thymocytes in each quadrant is indicated. H57 treatment produced a 49% reduction in viable thymocyte recovery in this experiment.

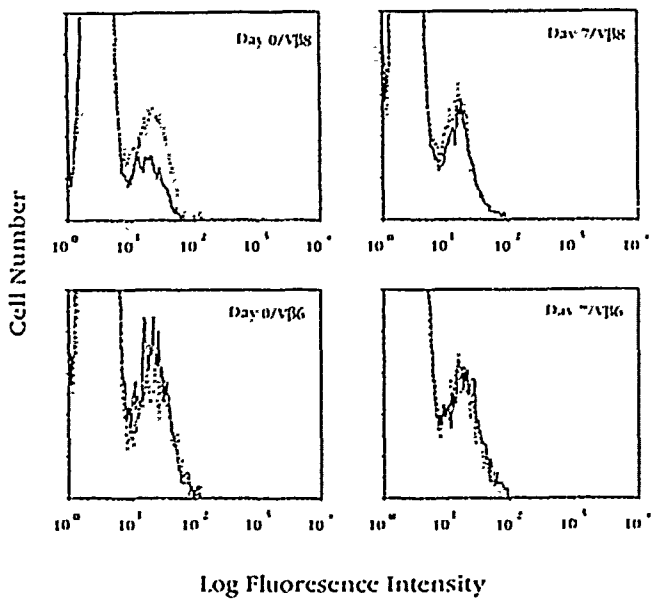

Figure 5. Thymocyte Vβ expression after exposure to SEB. Thymic lobes from B6 mice were harvested on day 0 or day 7 after conception and cultured in complete medium alone (· · · ·) or complete medium plus SEB at a final concentration of 30 μg/ml (——). After 24 h, thymocyte cell suspensions were prepared, cultured at 37°C for 8 h to allow re expression of modulated receptors (19), and stained with F23.1 or RR-4 7 culture supernatants followed by FITC-goat anti-mouse or FITC goat anti rat Ig, respectively. Control staining was done with the FITC labeled second antibody alone. The cells were analyzed as in Figure 3. The percentage of decrease in Vβ8⁺ thymocytes was 48% (day 0) and 17% (day 7), whereas the reciprocal increase in Vβ6⁺ thymocytes was 23% (day 0) and 9% (day 7).

with the data obtained from the in vivo injection of an anti-TCR antibody, indicate that thymocytes are more susceptible to activation-induced clonal deletion on the day of birth (and 3 weeks later) than 1 week after birth.

Induction of programmed cell death by TCR-independent reagents. To determine whether susceptibility to clonal deletion is dependent on the type of stimulus, thymocytes were tested for their response to two reagents that have been shown to cause thymocyte apoptosis, a corticosteroid, DEX, and a Ca^{2+} ionophore, ionomycin (4, 5, 25). In fact, the elevation of $[Ca^{2+}]_i$ that is caused by either TCR-mediated activation or ionomycin has been proposed as a critical event in activation-induced cell death (26). To quantify the survival of these cells after a 15-h culture, viability was determined by using flow cytometry to measure the exclusion of propidium iodide (Table II). Although thymocytes from day 0 mice were somewhat less viable in medium alone, the majority of cells from all groups survived in the absence of any stimulus. Thymocytes from mice of all ages were susceptible to killing by DEX and ionomycin, and with similar dose-response relationships. Analysis of parallel samples by agarose gel electrophoresis demonstrated that the treatments that caused increased propidium iodide uptake also resulted in the appearance of the typical DNA fragment "step ladder" that is characteristic of apoptosis

TABLE II

Thymocyte depletion by reagents that bypass the TCR[a]

Treatment	Concentration	Viable thymocytes (%)		
		Day 0	1 Week	3 Weeks
Medium		57 (100%)	74 (100%)	74 (100%)
DEX	10^{-9} M	49 (86%)	68 (92%)	68 (92%)
	10^{-8} M	23 (40%)	42 (57%)	39 (53%)
	10^{-7} M	12 (21%)	24 (32%)	27 (36%)
Ionomycin	0.1 μM	43 (75%)	62 (84%)	65 (88%)
	0.33 μM	26 (46%)	41 (55%)	44 (59%)
	1.0 μM	9 (16%)	10 (14%)	12 (16%)

[a] Thymic lobes from B6 mice on day 0 (day of birth) or at 1 or 3 weeks after birth were removed, cultured as a single-cell suspension for 15 h, and then analyzed for viability with a FACScan, as described in *Materials and Methods*. The data are expressed as the percentage of analyzed cells that did not stain with propidium iodide (viable). The numbers in parentheses represent (percentage of unstained cells in each treatment group/percentage of unstained cells in cultures with medium alone) × 100.

(data not shown) Thus, the deletionally refractory state of day 7 thymocytes does not appear to reflect changes in the endogenous autolytic machinery, because two well defined and independent means of inducing apoptosis were equally efficient in all groups.

Because of the recent observation that antigenic stimulation of T cell hybridomas that do not express the TCR-$\zeta\eta$ subset was ineffective at inducing programmed cell death (27), the examination of TCR-ζ and -η expression in neonatal thymocytes was undertaken. By using either S1 nuclease protection of mRNA or immunoblotting of anti-ζ-precipitable proteins with an anti ζ/η specific antiserum, we were unable to detect any marked differences in TCR-ζ_2 or TCR-$\zeta\eta$ expression between thymocytes from day 0 and 1-week- and 3-week old mice (data not shown).

DISCUSSION

The pattern of susceptibility of thymocytes to activation induced depletion in early life is remarkably similar to that for neonatal tolerance (10, 28, 29) and supports an important role for clonal deletion in this phenomenon. In the original studies, individual fetuses were injected with allogeneic cells in utero and challenged 6 to 8 weeks after birth with allogeneic skin grafts. It was found that, the earlier the injection, the better the tolerance. In fact, injection on or before fetal day 18 gave the best results, with injection after birth being progressively less effective. Because of the technical difficulty of injecting embryos, the phenomenon of neonatal tolerance has been studied almost exclusively in mice injected within the first 24 h of life, the relative lack of efficacy being offset by the injection of relatively large numbers of cells (30, 31). More recently, when Mls-1^a spleen cells were injected into Mls-1^b mice within 24 h of birth, it was found that cortisone-resistant or CD8$^+$ (mostly CD4$^-$ single-positive) Vβ6$^+$ thymocytes (i.e., those recognizing Mls-1^a) were depleted (32–34). In those studies, the relationship between deletion and developmental stage was not determined. The present study offers a quantitative and direct means of examining the phenomenon of neonatal tolerance at the cellular level. Two likely conclusions can be drawn from the data. First, neonatal tolerance and its unusual time course can be explained by the clonal deletion of immature, TCR^{low}, CD4$^+$ CD8$^+$ thymocytes, although it is possible that additional peripheral mechanisms also play a role. The difficulty in tolerizing with allogeneic cells 1 week after birth is that these thymocytes are relatively refractory to TCR-mediated deletion. Second, it is a paradox that, although one can easily delete thymocytes from 3-week-old mice with stimuli that directly bind the TCR, animals of this age cannot readily be tolerized with allogeneic cells. Therefore, the inability to tolerize young adult mice (3 to 6 weeks old) with allogeneic cells is not because their thymocytes have passed beyond a deletional state but is perhaps due to the presence of peripheral mature T cells that can mount a host vs-graft response that either prevents or masks the loss of alloreactive thymocytes. This is consistent with the observation that inoculation of 12-day old mice with allogeneic cells actually enhanced rejection of subsequent allogeneic skin grafts (30) and the finding that specific Vβ reductions in adult mice tolerized to Mls-1^b can occur if cyclophosphamide is administered to eliminate peripheral, Ag-reactive T cells (i.e., host-vs-graft effector cells) (35). Indeed, in other experiments, injection of SEB into young adult mice does not lead to the acute deletion of Vβ8$^+$ cells, whereas incubation of thymi from 5-week-old mice with SEB in an organ culture system does (our unpublished observations), supporting the notion that it is the complexity of the peripheral mature T cell response that prevents the experimentally induced clonal deletion of thymocytes from older mice.

The question of why the ability of TCR-mediated stimuli to cause clonal deletion varies in an alternating fashion after birth remains to be answered. An attractive hypothesis is that maturing thymocytes go through a stage at which they can be easily deleted and that fetal and early neonatal thymocytes represent a relatively synchronous wave of cells expressing this phenotype. Consistent with this possibility, discontinuous murine thymocyte growth and accumulation have been noted in the perinatal period (36). By 3 weeks after birth, new thymocyte precursors have presumably entered the thymus and, thereafter, at any given time, there will be asynchronously developing cells, among which a subset will be susceptible to clonal deletion. Based upon studies in organ culture, it has been suggested that there are two populations of $\alpha\beta$ TCR^{low} thymocytes, one susceptible to deletion by anti-$\alpha\beta$ and one resistant (19). If so, then in the relatively synchronized day 7 thymocytes the sensitive population may either have already been deleted or have matured to the resistant state. Regardless, the data demonstrate that all "immature" thymocytes (i.e., TCR^{low}, CD4$^+$ CD8$^+$) are not equally sensitive to TCR $\alpha\beta$-mediated clonal deletion and that the ratio of those that are susceptible to those that are resistant changes during development. Additional experiments examining other signaling pathways and/or changes in nuclear events will be required to elucidate the mechanisms that account for these phenotypes.

Acknowledgments. We are grateful to Juan Zuniga-Pflucker for help with the isolation of thymocytes, to Ralph Kubo for generously providing the H57 antibody, and to Ronald Germain and Dan Longo for critical review of this manuscript.

REFERENCES

1. Jenkins, J. K., and R. H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med. 165:302.*
2. Ramsdell, F., T. Lantz, and B. J. Fowlkes. 1989. A nondeletional mechanism of thymic self tolerance. *Science 246:1038.*
3. Blackman, M. A., H. Gerhard-Burgert, D. L. Woodland, E. Palmer, J. W. Kappler, and P. A. Marrack. 1990. A role for clonal inactivation in T cell tolerance to Mls-1^a. *Nature 345:540.*
4. Wyllie, A. H. 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature 284:555.*
5. Smith, C. A., G. T. Williams, R. Kingston, E. J. Jenkinson, and J. J. T. Owen. 1989 Antibodies to the CD3/T-cell receptor complex induce death by apoptosis in immature T-cells in thymic cultures. *Nature 337:181.*
6. Jenkinson, E. J., R. Kingston, C. A. Smith, G. T. Williams, and J. J Owen. 1989 Antigen induced apoptosis in developing T cells, a mechanism for negative selection of the T cell receptor repertoire. *Eur. J. Immunol. 19:2175.*
7. Mercep, M., P. D. Noguchi, and J. D. Ashwell. 1989. The cell cycle block and lysis of an activated T cell hybridoma are distinct processes with different Ca^{2+} requirements and sensitivity to cyclosporin A. *J. Immunol. 142:4085.*
8. Ucker, D., J. D. Ashwell, and G. Nickas. 1989. Activation-driven T cell death: requirements for de novo transcription and translation and association with genome fragmentation. *J. Immunol. 143:3461.*
9. Shi, Y., B. M. Sahai, and D. R. Green. 1989. Cyclosporin A inhibits

activation-induced cell death in T-cell hybridomas and thymocytes. *Nature 339:625.*
10. Billingham, R. E., L. Brent, and P. B. Medawar. 1953. Actively acquired tolerance of foreign cells. *Nature 172.603.*
11. Kubo, R. T., W. Born, J. W. Kappler, P. Marrack, and M. Pigeon. 1989. Characterization of a monoclonal antibody which detects all murine α/β T cell receptors. *J. Immunol. 142.2736.*
12. Staerz, U. D., H.-G. Rammensee, J. D. Benedetto, and M. J. Bevan. 1985. Characterization of a murine monoclonal antibody specific for an allotypic determinant on T cell antigen receptor. *J. Immunol. 134:3994.*
13. Kanagawa, O., E. Palmer, and J. Bill. 1989. A T cell receptor Vβ6 domain that imparts reactivity to the Mls^a antigen. *Cell. Immunol. 119:412.*
14. Leo, O., M. Foo, D. H. Sachs, L. E. Samelson, and J. A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA 84.1374.*
15. Kung, P. C., G. Goldstein, E. L. Reinherz, and S. F. Schlossman. 1979. Monoclonal antibodies defining distinctive human T cell surface antigens. *Science 206:347.*
16. Dialynas, D. P., Z. S. Guan, K. A. Wall, A. Pierres, J. Quintans, M. R. Loken, M. Pierres, and R. W. Fitch. 1983. Characterization of the murine T cell surface molecule, designated L3T4, identified by the monoclonal antibody GK1.5: similarity of L3T4 to the human Leu 3/T4 molecule. *J. Immunol. 131.2445.*
17. Saramiento, M., A. L. Glasebrook, and F. W. Fitch. 1980 IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt 2 antigen block T cell mediated cytolysis in the absence of complement. *J. Immunol. 125.2665.*
18. Zuniga-Pflucker, J. C., S. A. McCarthy, M. Weston, D. L. Longo, A. Singer, and A. M. Kruisbeek. 1989. Role of CD4 in thymocyte selection and maturation. *J. Exp. Med. 169.2085.*
19. Finkel, T. H., J. C. Cambier, R. T. Kubo, W. K. Born, P. Marrack, and J. W. Kappler. 1989. The thymus has two functionally distinct populations of immature $\alpha\beta^+$ T cells, one population is deleted by ligation of $\alpha\beta$TCR. *Cell 58:1047.*
20. Sussman, J. J., J. S. Bonifacino, J. Lippincott Schwartz, A. M. Weissman, T. Saito, R. D. Klausner, and J. D. Ashwell. 1988. Failure to synthesize the T cell CD3-ζ chain: structure and function of a partial T cell receptor complex. *Cell 52.85.*
21. Kappler, J., J. White, D. Wegmann, E. Mustain, and P. Marrack. 1982. Antigen presentation by Ia$^+$ B cell hybridomas to H 2 restricted T cell hybridomas. *Proc. Natl. Acad. Sci. USA 79:3604.*
22. Rabinovitch, P. S., and C. H. June. 1990. Measurement of intracellular ionized calcium and membrane potential. In *Flow Cytometry and Sorting.* Ed. 2. M. R. Melamed, T. Lindmo, and M. L. Mendelson, eds. Wiley-Liss, New York, pp 651-688.
23. White, J., A. Herman, A. M. Pullen, R. Kubo, J. W. Kappler, and P. Marrack. 1989. The Vβ-specific superantigen Staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell 56:27.*
24. Marrack, P., and J. W. Kappler. 1990. The Staphylococcal enterotoxins and their relatives. *Science 248.705.*
25. Wyllie, A. H., R. G. Morris, A. L. Smith, and D. Dunlop. 1984. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J. Pathol. 142:67.*
26. McConkey, D. J., P. Hartzell, J. F. Amador Perez, S. Orrenius, and M. Jondal. 1989. Calcium-dependent killing of immature thymocytes by stimulation via the CD3/T cell receptor complex. *J. Immunol. 143:1801.*
27. Mercep, M., A. M. Weissman, S. J. Frank, R. D. Klausner, and J. D. Ashwell. 1989. Activation driven programmed cell death and T cell receptor $\zeta\eta$ expression. *Science 246.1162.*
28. Billingham, R. E., L. Brent, and P. B. Medawar. 1955. Acquired tolerance of tissue homografts. *Ann. NY Acad. Sci. 59:409.*
29. Billingham, R. E., L. Brent, and P. B. Medawar. 1956 Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. *Philos. Trans. R. Soc. Lond. B Biol. Sci. 239.357.*
30. Brent, L., and G. Gowland. 1961. Cellular dose and age of host in the induction of tolerance. *Nature 192.1265.*
31. Billingham, R. E., V. Defendi, W. K. Silvers, and D. Steinmuller. 1962. Quantitative studies on the induction of tolerance of skin homografts and on runt disease in neonatal rats. *J. Natl. Cancer Inst. 28:365.*
32. MacDonald, H. R., T. Pedrazzini, R. Schneider, J. A. Lousi, R. M. Zinkernagel, and H. Hengartner. 1988. Intrathymic elimination of Mls^a-reactive Vβ6$^+$ cells during neonatal tolerance induction to Mls^a encoded antigens. *J. Exp. Med. 167.2005.*
33. Schneider, R., R. K. Lees, T. Pedrazzini, R. M. Zinkernagel, H. Hengartner, and H. R. MacDonald. 1989. Postnatal disappearance of self-reactive (Vβ6$^+$) cells from the thymus of Mls^a mice: implications for T cell development and autoimmunity. *J. Exp. Med. 169:2149.*
34. Speiser, D. E., R. Schneider, H. Hengartner, H. R. MacDonald, and R. M. Zinkernagel. 1989. Clonal deletion of self-reactive T cells in irradiation bone marrow chimeras and neonatally tolerant mice: evidence for intercellular transfer of Mls^a. *J. Exp. Med. 170.595.*
35. Eto, M., H. Mayumi, Y. Tomita, Y. Yoshikai, Y. Nishimura, and K. Nomoto. 1990. Sequential mechanisms of cyclophosphamide induced skin allograft tolerance including the intrathymic clonal deletion followed by the breakdown of the clonal deletion. *J. Immunol. 145:1303.*
36. Penit, C., and F. J. Vasseur. 1989. Cell proliferation and differentiation in the fetal and early postnatal mouse thymus. *J. Immunol. 142:3369.*